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Covalent Immobilization of EPCs-Affinity Peptide on Poly(L-Lactide-*co*-ε-Caprolactone) Copolymers to Enhance EPCs Adhesion and Retention for Tissue Engineering Applications

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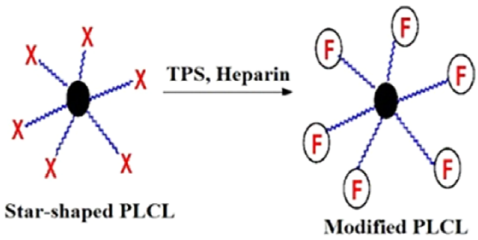
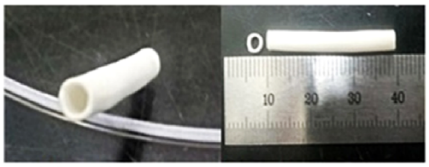
Abstract: Small diameter vascular grafts (inner diameter ≤ 6 mm) have a critical limitation regarding inner thrombotic reaction and occlude when implanted as artificial substitutes. *In situ* capture of endothelial progenitor cells (EPCs) could be beneficial to improve the endothelialization of artificial blood vessels. This study aimed to develop EPCs-affinity peptide (TPSLEQRTVYAK, TPS) and heparin-conjugated star-shaped poly(L-lactide-*co*-ε-caprolactone) (St-PLCL) copolymers to simultaneously capture EPCs and improve the hemocompatibility of vascular grafts, respectively. Electrospun membranes and small-diameter vascular grafts were fabricated by mixing linear PLCL, heparin-conjugated St-PLCL (PLCL-Hep), and TPS-conjugated St-PLCL (PLCL-TPS) copolymers. Vascular grafts exhibited biomechanical properties similar to the ISO standard. Membranes containing PLCL-Hep and PLCL-TPS showed fewer adhered platelets than did the control membranes. Moreover, electrospun membranes containing PLCL-Hep and PLCL-TPS adhered significantly to more EPCs than did the control group; however, three types of membranes did not appreciably differ in terms of the attachment of endothelial cells (ECs). Subcutaneous implantation of vascular grafts in Sprague-Dawley rats led to cellular infiltration and neotissue formation, which increased with the passage of time. Taken together, PLCL-TPS and PLCL-Hep copolymers can be fabricated into small-diameter vascular grafts to facilitate endothelialization through endogenous cell recruitment for vascular tissue regeneration applications.




Microfibrous vascular grafts containing TPS and heparin-conjugated PLCL

Keywords: poly(L-lactide-*co*-ε-caprolactone), vascular grafts, endothelial progenitor cells, cell adhesion, endothelialization, polyester, TPS.

1. Introduction

Cardiovascular diseases cause huge morbidities and mortalities world-wide. While autografts are gold-standard replacement option, limited numbers of appropriate donors or donor-site associated infection risks hamper their full utilization.[1] As an alternative, artificial blood vessels, such as, expanded polytetrafluoroethylene (ePTFE) and poly(ethylene terepthalate) (PET) have achieved success for vascular grafting, however, they often fail due to thrombosis, intimal hyperplasia, and infection, when used for grafting of small-caliber blood vessels.[2,3] Moreover, artificial blood vessels possess limited growth potential and require re-operation, especially in the pediatric patients, whose vessels continually change during the growth.[3] Consequently, biodegradable vascular grafts are being pursued, which could remodel within the host and disappear after the regeneration of an entirely new artery.[4,5]

Since biodegradable polymers are not bio-inert polymers, thrombotic reactions can occur easily. In addition, owing to the small size, even if only a small blood coagulation occurs, the inside of the blood vessel becomes blocked and the blood flow cannot pass through the vessel normally.[6] The most effective way to prevent a thrombotic reaction is endothelialization.[7] The lumen of blood vessels mainly consists of endothelial cells (ECs), which prevent platelets aggregation. Therefore, if we could mimic the inner surface of native blood vessels by attaching ECs in a synthetic graft, it may be advantageous for enhancing the patency of small-diameter vascular grafts. Various research groups have seeded ECs on the luminal side of artifi-

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cial vascular grafts, which improved the patency rate. However, this approach is hindered by the limited retention of ECs on the luminal side due to the circulation, the shortage of appropriate cell sources, and/or extensive *in vitro* cell manipulations.

To maximize the endothelialization, the use of endothelial progenitor cells (EPCs) has been proposed. EPCs are a type of adult stem cells, which are collectively referred to as intermediate cells from hematopoietic stem cells to ECs.[8] Since EPCs have stem cell like properties, they can proliferate and expand more than ECs. Therefore, when using *in situ* regeneration strategies, it is more effective to use EPCs rather than ECs, expecting EPC's proliferation and expansion in the body, and/or their differentiation into ECs.[9] A certain amount of EPCs is present in the peripheral circulation and various types of biomolecules have been used to capture EPCs *in situ*, including antibodies, aptamers, saccharides, magnetic molecules, and peptides.[10] Among these biomolecules, the 12-amino acids peptide "TPSLEQRTVYAK" (TPS) was discovered through phage-display method, which specifically captured EPCs and allowed the selective adhesion of EPCs when immobilized on scaffold materials.[11] Besides endothelialization, blood-contacting devices require hemocompatibility, which has been addressed by using antithrombogenic and/or nonbiofouling surface modifications.[12] Non-specific protein adsorption may also affect the hemocompatibility of vascular grafts and/or the function of the immobilized peptide and various types of molecules, such as, poly(ethylene glycol), hydrophobin, albumin, zwitterionic polymers, and heparin can prevent non-specific protein adsorption.[13-17] In addition, prevention of thromboembolic events is necessary for the long-term performance of small-diameter vascular grafts. Various biomolecules have been used to prevent thrombosis, such as hirudin and heparin. Heparin is a highly sulfated glycosaminoglycan and has been widely used as an anticoagulant in clinical settings for the prevention of thromboembolic diseases as well as in cardiac surgery. Heparin can be either covalently linked to polymers or physically trapped within scaffolds to prevent thrombosis and promote angiogenesis *in vivo*.[18]

Poly(L-lactide-*co*-ε-caprolactone) (PLCL) (50 : 50) is a mechano-elastic and biodegradable polymer, which exhibits mechanical properties similar to native blood vessels and therefore it may be advantageous to provide mechano-transduction to the recruited cells as well as it may inhibit graft failure caused by the mismatch of mechanical properties.[18-22] However, PLCL is a bio-inert polymer and needs biofunctionalization prior to *in vivo* implantation for the biocompatibility. Scaffold materials consisting of PLCL have been functionalized with various types of biomolecules using a variety of post-fabrication functionalization methodologies. While appealing, harsh functionalization procedures may compromise mechanical properties of scaffold materials. To cope with these limitations, we have designed star-shaped PLCL copolymers (St-PLCL), which could be bioactivated with biofunctional moieties in bulk to endow biocompatibility to unless otherwise inert polymeric materials.[23-26] Since St-PLCL copolymers would have more terminal hydroxyl groups than that of the linear PLCL copolymers, they may provide an ease for the incorporation of biofunctional molecules into scaffold materials.

We have reported the successful introduction of various types of peptides and biomolecules into St-PLCL copolymers, including neuropeptide substance P (SP), mesenchymal stem cell-affinity peptide (E7), and heparin, which enhanced cell adhesion, cellular infiltration, and tissue regeneration in scaffold materials.[23-27] Therefore, the aim of this study was to develop TPS and heparin-conjugated St-PLCL copolymers (PLCL-TPS and PLCL-Hep, respectively) for enhancing endothelialization and inhibiting thrombosis in artificial vascular grafts for *in situ* vascular tissue regeneration applications. We foresee that TPS peptide will play a role in specifically capturing EPCs, and heparin may support the role of the peptide by inhibiting the thrombosis of vascular grafts. TPS and heparin were conjugated with St-PLCL copolymers and used to fabricate electrospun membranes and microfibrous vascular grafts for *in vitro* and *in vivo* assays. *In vitro* platelet adhesion assay and cell adhesion assay were carried out by using electrospun membranes. EPCs and ECs were cultured on electrospun membranes with or without PLCL-TPS to elucidate the potential of TPS to specifically capture EPCs. Biomechanical properties of vascular grafts including water entry pressure, burst strength as well as tensile properties were evaluated. The initial biocompatibility of vascular grafts with or without TPS was evaluated by subcutaneous implantation in rats for up to 2 weeks and 4 weeks.

2. Experimental

2.1. Materials

L-Lactide was purchased from PURAC Biomaterials (Seoul, South Korea). Dimethyl-sulfoxide (DMSO), ε-caprolactone (ε-CL), 1-dodecanol, tripentaerythritol (TPE), formalin solution (10%, buffered), Dulbecco's phosphate buffered saline (D-PBS), tin(II)-bis(2-ethyl-hexanoate) [Sn(Oct)$_2$], dichloromethane (DCM), carbonyldiimidazole (CDI), poly(caprolactone) (PCL) (M_n, 80,000 Da), and heparin (Grade I-A, > 180USP units/mg) were purchased from Sigma Aldrich (Seoul, Korea). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was purchased from Tokyo Chemical Industry (Tokyo, Japan), and TPS peptide (TPSLEQRTVYAK-NH_2) (> 85% purity) was purchased from Peptron (Daejeon, South Korea). Chloroform ($CHCl_3$) and methanol (CH_3OH) were purchased from Daejung Chemical (Daejeon, South Korea).

2.2. Synthesis of linear and star-shaped PLCL copolymers

Linear and star-shaped PLCL (50:50) copolymers were synthesized by using ring-opening polymerization method following our previously reported method.[23] Briefly, 1-dodecanol and TPE were used as initiators to synthesize linear and St-PLCL copolymers. Polymerization was carried out in bulk in a 250 mL glass flask containing 100 mM L-lactide (LA), 100 mM ε-CL, 0.5 mM of 1-dodecanol, and 1.0 mM Sn(Oct$_2$). The flask was washed three times with nitrogen (N_2) and vacuumed for 6 h. The sealed flask was then transferred to a pre-heated silicone oil bath (150 °C) and polymerized for 24 h with magnetic stirring.

Thereafter, the produced copolymers were dissolved in $CHCl_3$, filtered through a 0.45 μm pore membrane, and precipitated in an excess amount of CH_3OH.

2.3. Conjugation of the TPS peptide and heparin with star-shaped PLCL copolymers

TPS peptide and heparin were conjugated with St-PLCL copolymers following our previously reported method.[23] Briefly, St-PLCL copolymer (M_n=71,252 Da and PDI=1.72, 4.26×10^{-5} moles) was dissolved in 10 mL of DCM with stirring under N_2 atmosphere for 2 h. When St-PLCL was dissolved, CDI (7.57×10^{-4} moles) was dissolved into 2 mL of anhydrous DCM and added into the St-PLCL solution, and the reaction was continued for 6 h under N_2. PLCL-CDI was precipitated using methanol and the filtered polymer was dried in a vacuum oven for 48 h. PLCL-CDI (1.61×10^{-5} moles) was dissolved in DCM and stirred for 3 h under N_2. For the synthesis of PLCL-TPS copolymers, TPS peptide (1.93×10^{-6} moles) was dissolved in DMSO and added into the stirring PLCL-CDI solution. The reaction was continued for 24 h at 25 °C.

PLCL-Hep copolymers were prepared by a direct-coupling reaction of the carboxylic (COOH) groups of the heparin and the hydroxyl (OH) groups of St-PLCL copolymers by using DCC/DMAP coupling chemistry as previously described.[27] Briefly, 200 mg of heparin was first dissolved in a mixture of formamide (15 mL) and dimethylformamide (15 mL) and stirred for 48 h at room temperature. DCC (0.01 g) and DMAP (0.006 g) were added to the heparin solution and stirred for 10 min. In a separate flask, 2.5 g of the St-PLCL (M_n=71,252 Da) was dissolved in 60 mL of methylene chloride and stirred for 6 h. PLCL solution was dropped into the heparin solution system and reacted at 50 °C for 12 h under a continuous stirring and an inert atmosphere. After the coupling reaction, the reaction system was concentrated and then precipitated using excess ethanol. After the precipitate was washed with distilled water, the precipitate was dissolved again using chloroform and the obtained solution was re-precipitated using excess ethanol. PLCL-TPS and PLCL-Hep copolymers were precipitated with ethanol, washed three times with PBS, and dried in a vacuum oven for 3 days.

2.4. Gel permeation chromatography

The average molecular weight of linear and St-PLCL copolymers were measured using a polystyrene calibrated μ-gel column GPC (Malvern, Viscotek GPCmaxVE 2001, Houston). Tetrahydrofuran was used as a mobile phase at 40 °C and a flow rate of 1.0 mL/min. Universal calibration was done with polystyrene standards with average molecular weights of 105,000 and 245,000 Da, respectively.

2.5. X-Ray photoelectron spectroscopy

The chemical composition of PLCL-TPS was investigated by using X-ray photoelectron spectroscopy (XPS, PHI 5800 ESCA system). A surface of about 50 Å was etched by the 3 kV argon sputtering for 0.5 min. Among the total binding energy spectrum, carbon, nitrogen, and oxygen were mainly analyzed.

2.6. Amino acid analysis

To evaluate the conjugation of TPS with St-PLCL copolymers, amino acid composition analysis was carried out according to previously published protocol.[20] Briefly, the conjugate sample was dissolved in $CHCl_3$ and then dried for hydrolysis. Sample was hydrolyzed in 6 N hydrochloric acid at 110 °C for 24 h, and then derived by phenylisothiocyanate (20 μL of methanol: water:triethylamine:phenylisothiocyanate in the ratio 7:1:1:1). After micro-centrifugation of the derived conjugate sample, supernatant was filtered with a 0.45 mm filter paper and analyzed by a high-pressure liquid chromatography (HPLC) equipped with a C18 column (Waters Nova-Pak C18, 3.9×300 mm, 4 μm), oven (46 °C), injector (HP 1100 series, Auto sampler), pump (HP 1100 series, binary pump), and variable wavelength detector (HP 1100 series). The solvent system (solvent A: 140 mM sodium acetate buffer, 0.15% triethylamine, 6% acetonitrile, 0.03% ethylenediaminetetraacetic acid (EDTA), pH 6.1 and solvent B: 60% acetonitrile, 0.015% EDTA) consisted of the linear gradient (0%-100%) of solvent B. Samples were detected at 254 nm at the flow rate of 0.4 mL/min and injection volumes of 2 μL for a standard curve, and 10 μL for samples, to determine the amounts of each amino acid. Mole fraction of each amino acid was calculated by comparing with the peak area from standard (250 pmol).

2.7. Toluidine blue assay

The heparin content in the star-shaped PLCL-Hep copolymers were quantified by using toluidine blue colorimetry assay.[27] Ten milligrams of a sample was added to 1 mL of a 0.2% sodium chloride solution (NaCl), and then toluidine blue solution (0.05% toluidine blue was added to a 0.01% aqueous solution of HCl (1,000 mL of 0.2% NaCl) was added. The absorbance of the aqueous layer was measured at 631 nm using a UV spectrophotometer (752 UV Grating Spectrophotometer, Shanghai). The heparin content was measured according to the standard curve of absorbance at 631 nm using a visible spectrophotometer.

2.8. Electrospinning for fabricating an artificial blood vessel

Three types of vascular grafts were fabricated by using electrospinning by mixing linear PLCL, PLCL-TPS, and PLCL-Hep

Table 1. Syntheses of membranes and vascular grafts by using electrospinning

Identification code	Linear PLCL[a]	PLCL-TPS	PLCL-Hep
Control	1400	-	-
TPS	1330	70	
TPS/Hep	1260	70	70

[a]Amount of linear PLCL, PLCL-TPS, and PLCL-Hep copolymers is given in mg. Control group consists of linear PLCL copolymers only. TPS group was fabricated by mixing linear PLCL and PLCL-TPS copolymers. TPS/Hep group consists of linear PLCL, PLCL-TPS, and PLCL-Hep copolymers.

copolymers (Table 1). Briefly, control group consists of linear PLCL copolymers (M_n=134,000 Da). TPS group consists of linear PLCL and PLCL-TPS copolymers. TPS/Hep group consists of linear PLCL, PLCL-TPS, and PLCL-Hep copolymers (Table 1). Briefly, 12% w/v polymer solution was made by using HFIP and the electrospinning conditions were as follows: voltage, 11 kV, needle size, 21G, the distance between the needle and collector, 18 cm, and the injection rate, 8.0 mL/h. The needle was fixed to the anode of the high voltage power supply and the cathode was connected to a rotating aluminum collector (outer diameter, 3.0 mm, 250 rpm). The solution was transferred to a high electric field grounded collector using a programmable pump (ESP 200 D, Nano NC). Electrospinning was continued until vascular grafts of approximately 500 μm thickness were achieved. Vascular grafts were vacuum-dried for 72 h. Microfibrous polycaprolactone (PCL) grafts were prepared by using following conditions: 25% w/v solution of polymer in a mixture of $CHCl_3$ and CH_3OH (5/1, v/v), needle-collector distance of 10 cm, flow rate of 8 mL/h, voltage of 10 kV, gold-coated stainless steel mandrel (OD=2.0 mm) (rpm=250 rpm), and a 21-guage needle (Table 1). Solution was delivered using a syringe pump (ESP200D, NanoNC).[28] For *in vitro* cell adhesion assay and platelet adhesion assay, electrospun membranes were fabricated by mixing 40 mg PLCL-TPS, 40 mg of PLCL-Hep, and 920 mg of linear PLCL. The electrospinning parameters used to produce membrane with 20-30 μm thickness were as follows: 9% w/v solution in HFIP, 20 cm needle-collector distance, 1 mL/h flow rate, 18 kV voltage, 21-gauge needle, and collector rotations (250 rpm).

2.9. Morphological analysis

The morphology of vascular graft was evaluated by using scanning electron microscopy (SEM; Hitachi, Tokyo, Japan) operated at 7.2 kV. Regarding the *in vitro* cell adhesion assay on electrospun membranes, cell-seeded membranes were fixed in 4% (v/v) formaldehyde overnight, dehydrated using graded ethanol, and dried. Gold coating was used to increase the conductivity for SEM imaging through the 'IB3 sputter-coater model' (Eiko, Hitachinaka, Japan). The average diameter of microfibers was measured from at least 100 fibers and reported.

2.10. Mechanical properties of vascular grafts

Mechanical properties of vascular grafts (n=5) were measured by using a tensile testing machine (Instron 5988, USA) following ISO 7198 recommendation of testing of vascular graft at a strain rate of 100 mm/min. Burst pressure was measured with a self-made instrument by filling a graft of 1.0 cm in length (n=5) with Vaseline, fastening one end and hermetically closing the other end to a vascular graft. A constant filling rate of 0.1 mL/min was applied, and the filling pressure was recorded until the graft was burst. Graft were tested and the average results are reported. Water entry pressure was measured by injecting water into vascular grafts. One side of the graft was immediately tied, and the graft was filled with distilled water at a pressure of 0.5 psi for 10 s, then the water pressure was increased gradually until the first leakage was observed on the graft wall. The porosity of vascular grafts was measured by using a mercury-intrusion porosity meter (Auto pore IV 9500, Micromeritics, Georgia, U.S.A).

2.11. *In vitro* platelet adhesion

To test the platelet adhesion and activation, control or TPS/Hep membranes of appropriate size were placed in 12-well cell culture plates (n=5).[29,30] To each well, 300 μL of human platelet rich plasma was added and the samples were incubated at 37 °C for 1 h. The unattached platelets were removed by rinsing 3 times with PBS, and the samples were characterized for platelet adhesion and activation. For the adhesion assay, the samples were fixed with 2.5% glutaraldehyde and dehydrated with gradient ethanol. Platelet adhesion to the sample surface was observed by SEM, and the quantitative analysis was performed.

2.12. *In vitro* cell adhesion assay

Mononuclear EPCs were purchased from ZenBio (NC, USA). Cells were separated from the human umbilical cord blood using density gradient centrifugation method. We cultured mononuclear cells in endothelial growth basal medium-2 (EBM-2; Lonza, Basel, Switzerland) and endothelial cell growth supplements (SingleQuots™ Kit; Lonza). Ten days after the culture, colonies with pebble form (EPCs) were selected and expanded to multiple passages. Cultured EPCs were identified by immunostaining CD31, CD34, vWF antibodies, and the tube formation test.[15] Morphologically, EPCs have a more widespread morphology than that of ECs. HUVECs were kindly granted by the Research and Development Institute, MCTT, Seoul National University of Technology, South Korea and were maintained in EBM-2. Cells were cultured for at least a week in a humidified atmosphere (95% air, 5% CO_2, and 37 °C before seeding experiments and were used below passage 6.

Control, TPS, and TPS/Hep samples (n=5 per group) were punched from electrospun membranes and placed into 24-well tissue culture plate and sterilized by ultraviolet radiation (UV) for up to 30 min. EPCs or ECs suspensions (5×10^4 cells/mL) were dropped onto membranes. After incubation for 2 h, the medium was aspirated and the loosely-adhered cells were washed three times with PBS. Membranes were fixed with 4% paraformaldehyde and stained with 10 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI). Test specimens were observed under a confocal laser scanning microscope (ZEISS LSM 710). The numbers of cells attached to the membranes were quantified from six randomly chosen fields (10×magnification).

Cell viability was evaluated using a live/dead cell assay kit (Life Technologies, USA) following the manufacturer's instructions. Control, TPS, and TPS/Hep membranes were coated with collagen type 1C following our previous published method.[25] Cell suspension (HUVECs, 1.2×10^4 cells) was added onto the membranes and the plate was incubated at 37 °C with 5% CO_2 for 24 h. After washing with PBS, the live/dead cell assay was performed. Calcein A (5 μL) and Ethidium homodimer-1 (20

μL) were added to 10 mL of PBS and mixed. About 500 μL of this solution was added per well, ensuring that the samples were wet. The plate was incubated at 37 °C with 5% CO_2 for 45 min. Images were captured by confocal laser scanning microscope (ZEISS LSM 710).

2.13. Evaluation of biocompatibility

All animals were treated in accordance with the recommendations for the handling of biomedical research laboratory animals, summarized by the Korea Institute of Science & Technology and the Committee on Safety and Ethical Handling of Laboratory Experiments by the Seoul National University. Sprague-Dawley rats (male, 7 weeks old, body weight 200-250 g, Orient Bio INC, Gyeonggi-do, Korea) received either control or TPS/Hep grafts subcutaneously (n=5 per group) for up to 2 weeks and 4 weeks. The animals were anesthetized with isoflurane (1% for induction 2% for maintenance) and anesthetized with 5 mg/100 g of ketamine IM. Under aseptic conditions, a muscular pouch was made using dull scissors, without damaging the deep muscles, by vertical incision on the posterior skin of the rats. The grafts were implanted in the space between the skin and the deep muscles and the skin was sutured using a 3.0 cm silk suture. After 2 or 4 weeks, the animals were sacrificed and samples were explanted. The samples were fixed in 10% formalin for 24 h and embedded into a mixture of paraffin and ethylene vinyl acetate (5:1). For hematoxylin and eosin (H&E) staining, the paraffin blocks were cut into 6 μm thick slices.

2.14. Statistical analysis

Single comparisons were made by using two-tailed student's t-test. Multiple comparisons were performed using a one-way ANOVA and Tukey's post-hoc analysis through Origin Pro 9 software (OriginLab, USA). Significance level was accepted at a P-value below 0.05. Data are expressed as the mean±standard error of mean.

3. Results and discussion

3.1. Material characterization

Linear and St-PLCL copolymers were synthesized by using ring-opening polymerization method. The number-average molecular weight (M_n) of the linear and St-PLCL copolymers was measured by GPC and found to be 134,000 Da and 71,252 Da, respectively. Heparin was conjugated with St-PLCL copolymers through esterification by reacting the hydroxyl groups of St-PLCL copolymers and the carboxylic groups (-COOH) of hep-

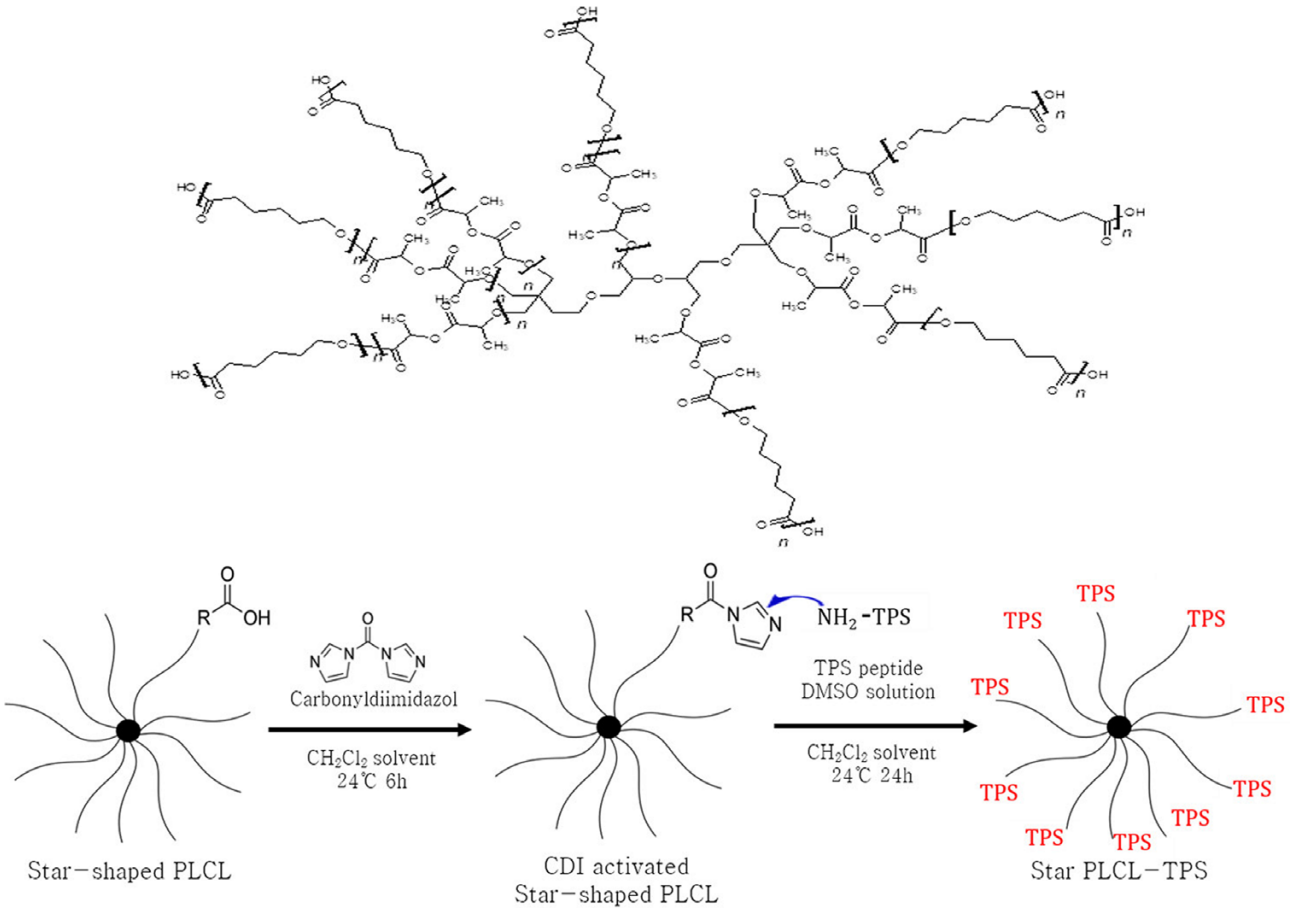

Figure 1. Schematic illustration of St-PLCL copolymers and PLCL-TPS copolymers. St-PLCL copolymers exhibit more terminal moieties than that of the linear PLCL copolymers. St-PLCL-TPS copolymers were synthesized by activating the hydroxyl functional groups of St-PLCL copolymers with the CDI. Amino groups of TPS were reacted with the activated functional groups of St-PLCL copolymers.

arin by using DCC/DMAP coupling method (Figure 1). TPS was conjugated with St-PLCL copolymers by reacting the amino groups of the peptide with the activated hydroxyl groups of St-PLCL copolymers (Figure 1). Electrospun membranes and vascular grafts were fabricated by using electrospinning (Figure 2). Heparin content of PLCL-Hep copolymers were determined by using toluidine blue assay and found to be 0.16 μg per mg of St-PLCL copolymers.

XPS elemental analysis was used to confirm the conjugation of TPS with St-PLCL copolymers (Figure 3). All XPS spectra reveal that C and O are predominant species and they occur at 285 and 532 eV, respectively. The C1s peaks corresponding to different bonds appeared in the range of 284 to 290 eV (C-C/C-H, 285 eV; C-O, 286.7 eV; C=O, 288.2-3 eV; and O-C=O, 289.2-

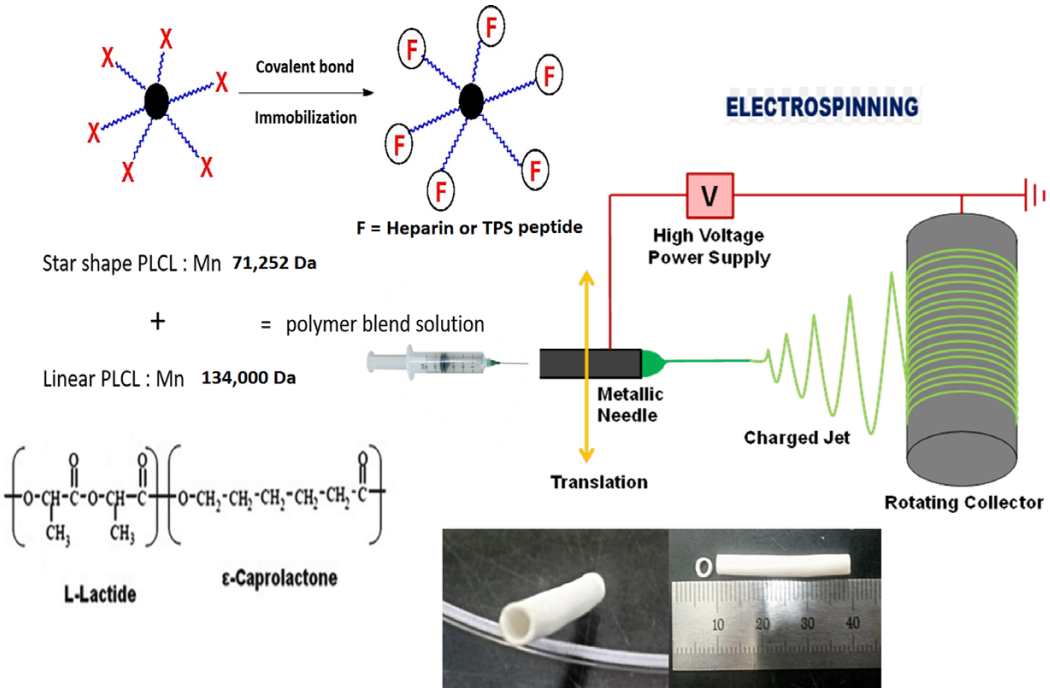

Figure 2. Fabrication of vascular grafts by using electrospinning. Small-diameter vascular grafts (inner diameter, 3.0 mm and graft wall, 500 μm) were fabricated by mixing the appropriate proportions of linear PLCL copolymers, PLCL-TPS copolymers, and PLCL-Hep copolymers.

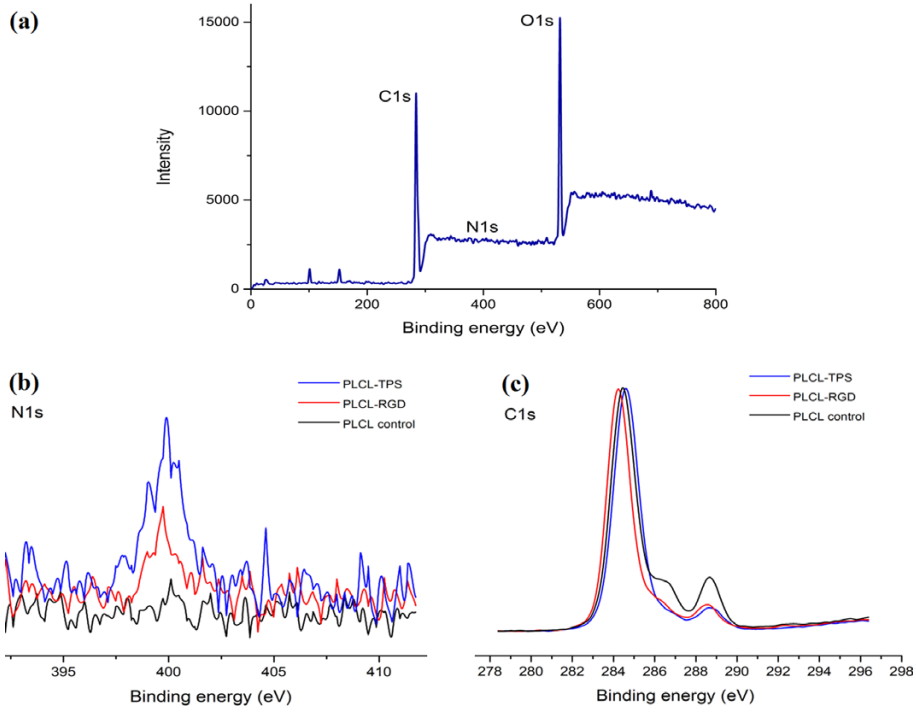

Figure 3. XPS analysis was carried out to evaluate the conjugation of TPS with St-PLCL copolymers. PLCL-RGD copolymers were synthesized as a reference. PLCL-TPS and PLCL-RGD copolymers revealed the presence of a peak corresponding to the nitrogen.

9 eV).[31] Since a peptide is composed of a repetition of amide bonds that contain nitrogen (N), the presence of the peptide in PLCL-TPS copolymers can be confirmed through elemental analysis for 'N'. The general value of the XPS nitrogen peak was present at 400 eV and this peak appeared in the star-shaped PLCL-TPS copolymers (Figure 3). In addition, as a positive control, the widely known RGD peptide was analyzed, which also showed a peak at 400 eV. Since the N-terminal of the peptide is attached to the activated hydroxyl groups of St-PLCL copolymers, the 289 eV portion corresponding to the C1s is reduced when the peptide is attached. Therefore, we could indirectly confirm that peptides are attached to the St-PLCL copolymers. TPS content were determined by using amino acid analysis and found to be 384.23 nmol per mg of PLCL-TPS copolymers. The conjugation method of TPS and heparin is facile and can also be extended to other peptides or polymeric materials for the introduction of bioactive moieties into scaffold materials. Whereas scaffold materials can be functionalized with bioactive moieties post-fabrication, harsh modification conditions may compromise the mechanical properties of scaffold materials. On the other hand, we introduced TPS and heparin into St-PLCL copolymers in bulk and therefore the modified copolymers can be fabricated into different shapes and structures as needed for tissue engineering applications.

3.2. Fabrication of vascular grafts and evaluation of mechanical properties

Microfibrous PLCL grafts (wall thickness, 500 μm and inner diameter, 3.0 mm) were fabricated by using electrospinning and their morphology was discerned through SEM (Figure 4, Table 1). The average diameter of microfibers was found to be 5.5±0.8 μm. The porosity and pore size distribution of vascular grafts was measured by using mercury-intrusion porosity meter. The average pore size was found to be 35.2 μm and pore sizes were in the range of 20-40 μm. The measured porosity was 46.5±5.6%. Mechanical properties of PCL and PLCL vascular grafts including water entry pressure, burst strength, young's modulus, and elastic recovery were evaluated and the obtained results are summarized in Figure 5 and Table 2. Microfibrous PCL grafts were prepared as a reference as they have been used to induce better cellular infiltration and neotissue regeneration than that of the nanofibrous vascular grafts.[28] The water entry pressure of PLCL grafts was found to be 3.90±0.23 psi, which was higher than that of the ISO standard value (3.5±0.0 psi). On the other, the water entry pressure of PCL grafts was found to be 1.3±0.12 psi. Similarly, the burst strength of PLCL grafts was found to be 72.5±4.62 psi, which was higher than that of the ISO standard (18.0±0.0 psi) and was comparable to the PCL grafts (61.7±3.2). The tensile strength of PCL and PLCL grafts was slightly higher than that of the ISO standard (ISO standard value, 600±0.0 MPa; PLCL, 702.44±31.3 MPa; and PCL, 712.38±24.9 MPa) and both types of grafts exhibited almost similar value of tensile strength. In elastic recovery tests, PLCL grafts showed good elasticity and softness and recovered to 90% of their original length, even their length was increased five times to their original length. On the other hand, the recovery of PCL graft was only 50% for only 10% elongation. Therefore, the mechano-elastic PLCL copolymer may be advantageous for vascular tissue regeneration.[14]

Whereas electrospun scaffold materials exhibit nanofibrous morphology, they are often hindered with the limited cellular

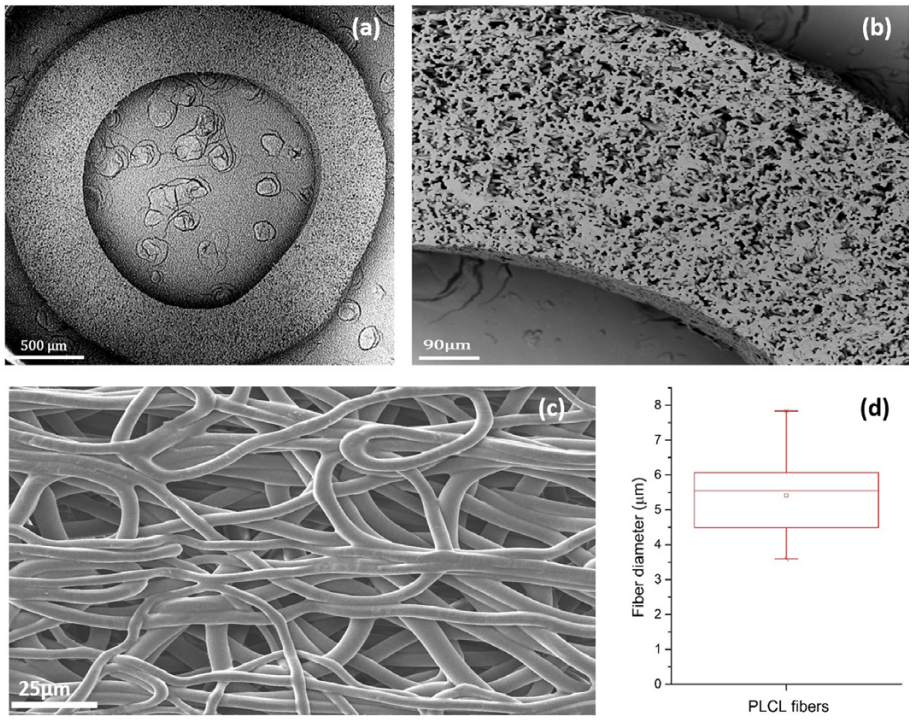

Figure 4. Morphological analysis of vascular grafts. (a) Whole image, (b) cross section, (c) surface, and (d) fiber diameter. Microfibrous vascular grafts were fabricated to enhance cellular infiltration and neotissue regeneration. Scale bars have been shown on the images.

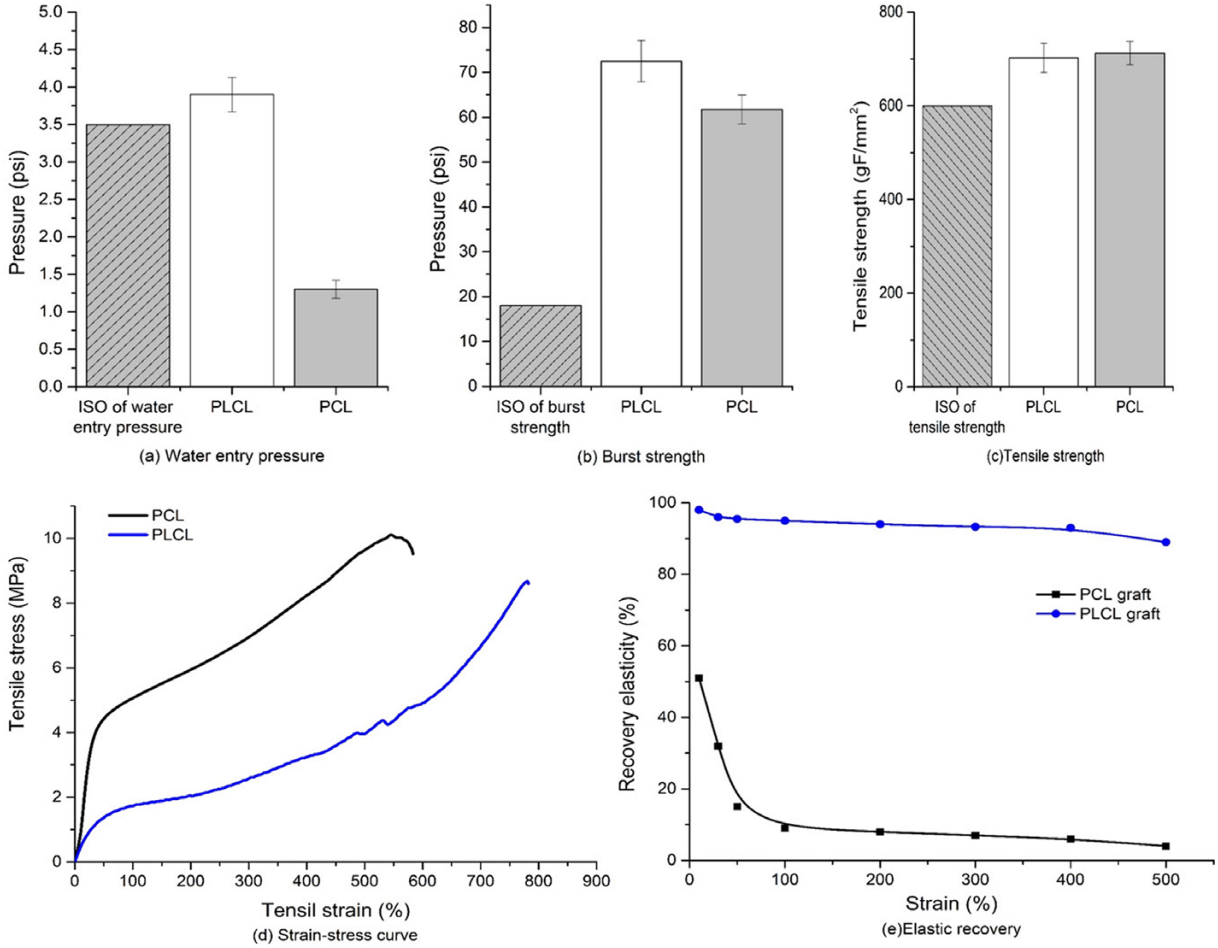

Figure 5. Mechanical properties of PCL and PLCL vascular graft (n=5 per group). Water entry pressure, burst strength, tensile strength, strain-stress curve, and elastic recovery of PCL and PLCL grafts.

Table 2. Evaluation of mechanical properties of polycaprolactone (PCL) and poly(L-lactide-*co*-ε-caprolactone) (PLCL)-based vascular grafts

Sample	Young's modulus (MPa)	Strain at rupture (%)	Maximum stress (MPa)	Maximum load (N)
PLCL graft	4.49	492	10.11	19.05
PCL graft	2.41	787	8.67	16.36

infiltration and less tissue regeneration. Consequently, researchers have attempted to fabricate scaffold materials with thick fibers and large pores to afford better cellular infiltration and neotissue regeneration than that of the scaffold materials containing thin fibers and small pores. Kong's group has elucidated that microfibrous grafts not only promote cellular infiltration and neotissue formation into vascular grafts, but also facilitate macrophages polarization toward anti-inflammatory (M2) macrophages phenotypes, which then promotes tissue regeneration.[28] Therefore, we fabricated microfibrous vascular grafts in this study.

Similarly, various research groups have reported successful vascular regeneration by using PCL-based small-diameter vascular grafts. However, the mismatch of mechanical properties of PCL grafts and the native blood vessels may lead to the intimal hyperplasia. On the other hand, mechano-elastic PLCL grafts exhibit mechanical properties similar to that of the native blood vessels and therefore they may offer an advantage over PCL grafts and may not lead to the mismatch of mechanical properties after implantation. In addition, being mechano-elastic, PLCL grafts may induce mechano-transduction and enhance the regeneration of vascular cell types. Indeed, we found better SMCs regeneration in the graft wall in PLCL grafts than that of the PCL grafts (data not shown).

3.3. Platelet adhesion assay

Since we incorporated PLCL-Hep into electrospun membranes and vascular grafts, we carried out *in vitro* platelet adhesion assay and the obtained results are shown in Figure 6. Electrospun membranes containing PLCL-TPS and PLCL-Hep (TPS/Hep group) showed significantly lower numbers of platelets (4.62×10^5/cm^2) than that of the control group (0.76×10^5/cm^2). The less numbers of adhered platelets are attributed to the heparin, which may also be advantageous for inhibiting the thrombosis of the vascular grafts. Shafiq *et al.* have also observed less

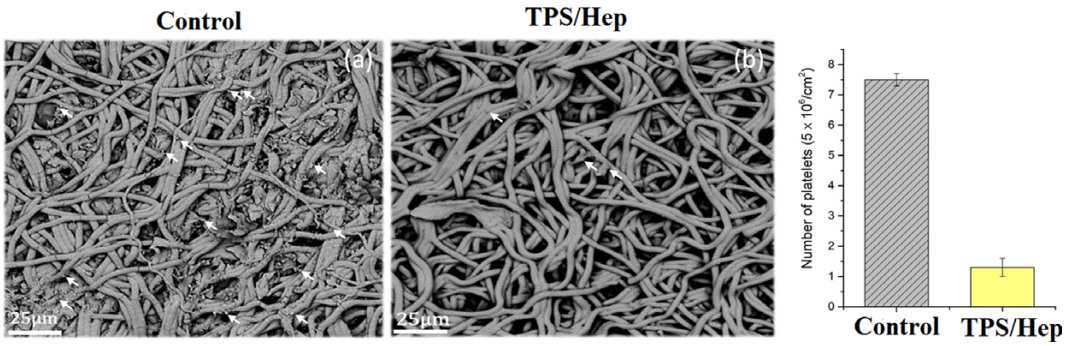

Figure 6. Platelet adhesion assay (n=5 per group). (a) PLCL, (b) TPS/Hep group. Quantitative analysis revealed significantly less platelet adhesion in the TPS/Hep membranes than that of the control group. Scale bar, 25.0 μm.

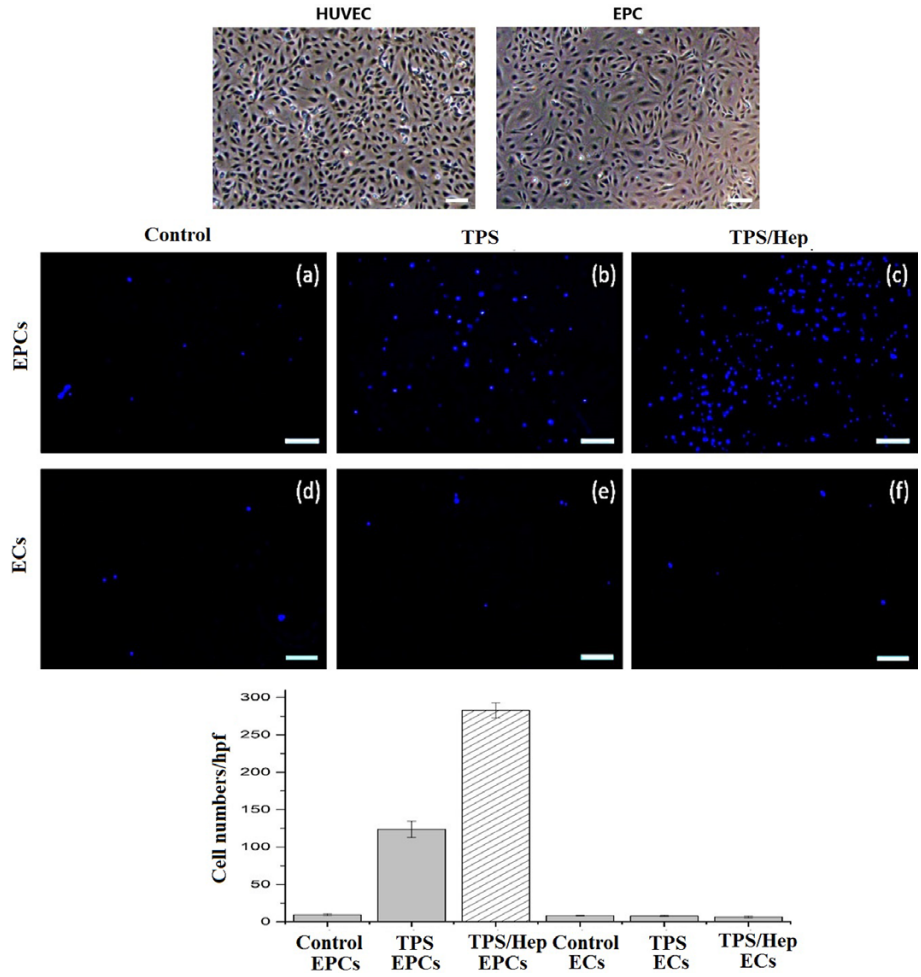

Figure 7. *In vitro* cell adhesion assay (n=5 per group). EPCs (a-c) and ECs (d-f) were seeded on control, TPS, or TPS/Hep membranes. Morphology of EPCs and HUVECs has been shown as a reference. TPS and TPS/Hep membranes showed significantly higher numbers of EPCs than that of the control group. Of TPS and TPS/Hep groups, later exhibited significantly higher numbers of EPCs. On the other hand, three groups did not significantly differ in terms of the adhesion of ECs. Scale bar is 300 μm.

numbers of adhered platelets in vascular grafts containing heparin.[23,26] Similarly, Park's group has reported improved hemocompatibility of heparin-conjugated poly(L-lactide) (PLLA).[29,30]

3.4. Adhesion of EPCs and ECs

Since TPS has been reported to selectively bind to EPCs, we carried an *in vitro* EPCs attachment assay to evaluate the potential of PLCL-TPS copolymers to specifically capture EPCs. Membranes containing TPS (TPS group) showed significantly higher numbers of attached EPCs than that of the control group (Control, 9.3±1.21 and TPS, 123.7±11.1 numbers of EPCs per high power field, hpf) (Figure 7). Furthermore, membranes containing TPS and heparin (TPS/Hep group) showed significantly higher numbers of EPCs than that of the TPS only group (TPS/Hep, 282.7±9.85 numbers of EPCs per hpf) (Figure 7). On the

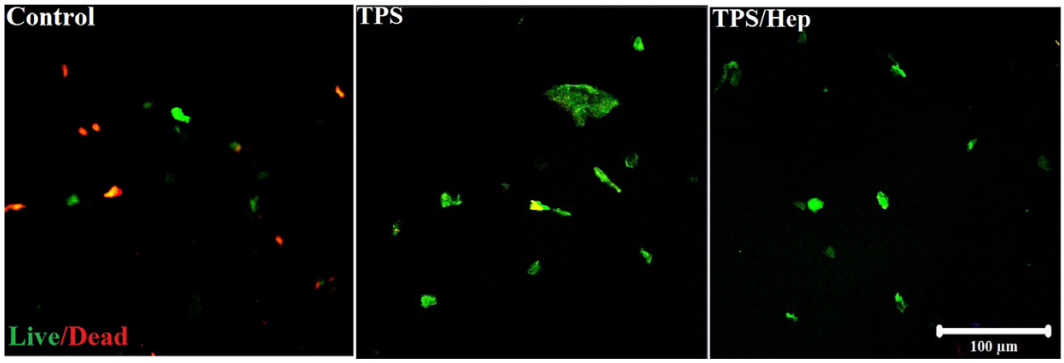

Figure 8. Determination of cell viability on control, TPS, and TPS/Hep membranes. Live and dead cell assay (Calcine AM/green/live cells; Live/dead cells; Ethidium homodimer/red/dead cells) after 24 h culture on PLCL and PLCL/PLCL-E7 meshes. Scale bar, 100 μm.

other hand, control, TPS, and TPS/Hep groups did not significantly differ in terms of the attachment of HUVECs and showed the adhesion of a few numbers of HUVECs (Control, 8.2±0.5; TPS, 7.9±0.78; and TPS/Hep, 6.5±1.1 cells per hpf) (Figure 7). Noticeably, we evaluated adhesion of EPCs and ECs on uncoated electrospun membranes.

To prove the stable adhesion and long-term viability of ECs on electrospun membranes, we coated control, TPS, and TPS/Hep membranes with collagen type 1c and seeded HUVECs on membranes for up to 24 h. Afterwards, we carried out live/dead assay, which revealed adhesion of HUVECs in control, TPS, and TPS/Hep membranes (Figure 8). Control group showed only few numbers of live cells. In contrast, TPS and TPS/Hep groups revealed the presence of more numbers of live cells than that of the control group. Therefore, we believe that ECs differentiated from EPCs can stably attach the electrospun membranes or vascular grafts. Parralely, we think that the introduction of a motif capable of adhering ECs will be help-

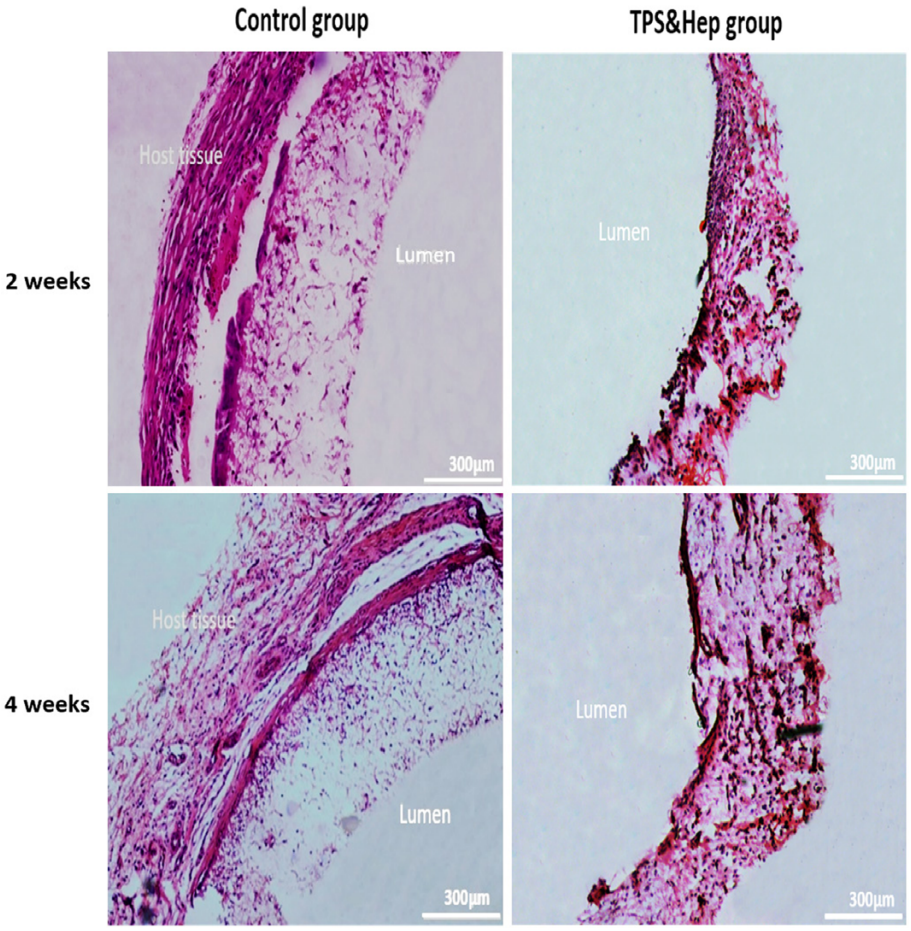

Figure 9. Evaluation of biocompatibility of control and TPS/Hep grafts after subcutaneous implantation in rats for up to 2 weeks and 4 weeks (n=5 per group per time point). Both types of vascular grafts favored cellular infiltration and neotissue formation in the graft wall. Scale bars have been shown on the images.

ful for the retention of host-recruited and/or EPCs-differentiated ECs. Since non-specific protein adsorption may compromise the function of TPS, thus lowering its ability to effectively promote cellular adhesion, heparin may induce repulsive forces to proteins. Indeed, TPS/Hep group has shown higher numbers of adhered EPCs than that of the TPS only group.[13-17]

TPS was discovered through phage-display method and shown to specifically capture EPCs. Various biomaterials have been designed to introduce TPS into scaffold materials including polypeptides and hydrophobins. Our strategy involves the fabrication of vascular grafts for *in situ* blood vessel regeneration through the recruitment of endogenous stem/progenitor cells. In our previous studies, we have developed neuropeptide substance P conjugated St-PLCL copolymers, which showed endogenous recruitment of stem cells and also led to the infiltration of more numbers of host cells.[26] Similarly, we developed stromal cell-derived factor 1 alpha (SDF-1α) peptide-tethered St-PLCL copolymers, which showed enhance cellularization and neotissue formation *in vivo.*[23] Since vascular grafts often fail due to limited endothelialization, we attempted to enhance *in situ* endothelialization by specifically capturing EPCs from the peripheral circulation, which may differentiate into ECs and enhance the endothelium regeneration into cell-free vascular grafts. On the other hand, PLCL-Hep copolymers were incorporated to improve the hemocompatibility and overcome the possible inactivity of TPS in the presence of plasma proteins. Therefore, our material approach can be advantageous to simultaneously enhance endothelization and vascular regeneration in cell-free vascular grafts.

3.5. Evaluation of biocompatibility

The initial biocompatibility of PLCL vascular grafts containing TPS and heparin was evaluated in a subcutaneous implantation model in rats for up to 2 weeks and 4 weeks (Figure 9). Implanted grafts were evaluated by using H&E staining. Generally, the cellularization in the graft wall increased in both types of vascular grafts with time. TPS/Hep grafts exhibit higher numbers of cells and neotissue regeneration than that of the control grafts 2 weeks and 4 weeks after implantation.

Our study also has certain limitations. First, we evaluated EPCs and ECs adhesion on electrospun membranes containing PLCL-TPS and PLCL-Hep under static conditions. The binding affinity of electrospun membranes to EPCs and ECs may be different under dynamic conditions in a bioreactor *in vitro* or *in vivo*. Second, we implanted vascular grafts subcutaneously, which did not replicate the actual implantation microenvironment. Therefore, the evaluation of vascular grafts with PLCL-TPS in a real implantation model is warranted. Third, we did not evaluate protein adsorption on electrospun membranes containing TPS and heparin. Nonetheless, we could observe specific capture of EPCs in TPS and TPS/Hep groups as well as the highest numbers of EPCs in TPS/Hep membranes, which may be beneficial for enhancing endothelialization *in vivo*. Furthermore, TPS and Hep-conjugated St-PLCL copolymers can be fabricated into different shapes and structures as needed for tissue engineering applications.

4. Conclusions

Star-shaped PLCL copolymers were functionalized with TPS and heparin to promote *in situ* endothelialization through the capture of EPCs from the peripheral circulation. XPS analysis, amino acid analysis, and toluidine blue assays demonstrated successful conjugation of TPS and heparin with the St-PLCL copolymers. PLCL-based vascular grafts containing PLCL-TPS and PLCL-Hep copolymers were fabricated by using electrospinning, which exhibited microfibrous morphology and showed sufficient biomechanical properties. *In vitro* assays demonstrated an inhibition of platelet adhesion and an increment in the EPCs capture resulted by PLCL-TPS and PLCL-Hep copolymers. However, control, TPS, and TPS/heparin groups did not appreciably differ in terms of the adhesion of ECs. Subcutaneous implantation of vascular grafts revealed their potential to enhance cellularization and promote tissue regeneration *in vivo.* Taken together, this methodology can be extended to develop cell-free vascular grafts for *in situ* tissue regeneration.

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